Portable smartphone quantitation of prostate specific antigen (PSA) in a fluoropolymer microfluidic device

Biosensors and Bioelectronics 70 (2015) 5–14

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Portable smartphone quantitation of prostate specific antigen (PSA) in a fluoropolymer microfluidic device

Ana I. Barbosa [a], Poonam Gehlot [a], Kalpita Sidapra [a], Alexander D. Edwards [b,c], Nuno M. Reis [a,c,*]

[a] Department of Chemical Engineering, Loughborough University, Loughborough LE11 3TU, United Kingdom
[b] Reading School of Pharmacy, Whiteknights, PO Box 224, Reading RG6 6AD, United Kingdom
[c] Capillary Film Technology Ltd, Daux Road, Billingshurst, West Sussex RH14 9SJ, United Kingdom

ARTICLE INFO

Article history:
Received 7 January 2015
Received in revised form
2 March 2015
Accepted 3 March 2015
Available online 5 March 2015

Keywords:
Microfluidic
Miniaturised ELISA
Smartphone readout
Fluorescence
Colorimetric
Point of care diagnostics

ABSTRACT

We present a new, power-free and flexible detection system named MCFphone for portable colorimetric and fluorescence quantitative sandwich immunoassay detection of prostate specific antigen (PSA). The MCFphone is composed by a smartphone integrated with a magnifying lens, a simple light source and a miniaturised immunoassay platform, the Microcapillary Film (MCF). The excellent transparency and flat geometry of fluoropolymer MCF allowed quantitation of PSA in the range 0.9 to 60 ng/ml with < 7% precision in 13 min using enzymatic amplification and a chromogenic substrate. The lower limit of detection was further improved from 0.4 to 0.08 ng/ml in whole blood samples with the use of a fluorescence substrate. The MCFphone has shown capable of performing rapid (13 to 22 min total assay time) colorimetric quantitative and highly sensitive fluorescence tests with good %Recovery, which represents a major step in the integration of a new generation of inexpensive and portable microfluidic devices with commercial immunoassay reagents and off-the-shelf smartphone technology.

1. Introduction

Decentralisation of diagnostic testing to near the patient sites is both a trend and a need in clinical diagnostics. Immunoassay platforms (the most common laboratory bioanalytical tool) and detection systems must therefore be adapted for point of care (POC) testing, which requires the ability to design affordable, portable, and user-friendly immunoassay systems capable of rapid and sensitive detection using well established immunoassay chemistries (von Lode, 2005).

Diagnostic tests are routinely used to diagnose and select treatment options for many critical health conditions, including cardiovascular diseases, sepsis, ovarian and prostate cancer, demand quantitation of one or multiple analytes (Altintas et al., 2014; Coelho and Martins, 2012; Loeb and Catalona, 2007; Zhang et al., 2011). Agglutination and lateral flow assays are the most widely used POC immunoassay tests, however these formats are usually qualitative or semi-quantitative, lacking both the sensitivity for many important biomarkers and the ability to perform multiplex analysis (von Lode, 2005). This has driven the development of microfluidic immunoassay platforms (Gervais et al., 2011), combining minimal diffusion distances and high surface-area-to-volume ratios for improved performance, with the use microchannels or beads for rapid and sensitive detection of analytes from small sample volumes (Gervais and Delamarche, 2009; Ikami et al., 2010; Park et al., 2012).

Optical detection is often preferred in POC testing, as it can rapidly and simply provide high-resolution microscopic and macroscopic information (Pierce et al., 2014; Zhu et al., 2013). The recent fall in cost of optoelectronic components now offers cost benefits for portable detection systems. Two most common optical detection techniques used in microfluidic immunoassays are fluorescence and chemiluminescence, due to their excellent sensitivity (Lin et al., 2010). However, the readout equipment used to detect these signals is complex and expensive, typically requiring a fluorescence or confocal microscope or high-sensitivity optical sensors, and therefore not portable or cost-effective for POC use (Myers and Lee, 2008). The opportunity for using simple portable optical detection in microfluidic diagnostics has recently arisen because of the rapid expansion in consumer electronics such as high-performance smartphones cameras that are now ubiquitous, and have driven down the price of high performance digital image sensors combined with portable computers (Zhu et al., 2013). Smartphones are portable, widely available, user-friendly and low cost, and are therefore suitable for integration into a microfluidic

* Corresponding author at: Department of Chemical Engineering, Loughborough University, Loughborough, LE11 3TU, United Kingdom. Fax: +44 0 1509 223 923.
E-mail address: n.m.reis@lboro.ac.uk (N.M. Reis).

http://dx.doi.org/10.1016/j.bios.2015.03.006

platform for POC diagnostics.

Examples of microfluidic diagnostic tests based on smartphone measurement include colorimetric detection of *Salmonella* from an immunoagglutination assay (Park et al., 2013); measurement of urine, saliva and sweat pH (Erickson et al., 2014; Shen et al., 2012); quantitation of vitamin D measured using a competitive lateral flow immunoassay (Lee et al., 2014); and prostate specific antigen (PSA) quantitation from a sandwich microfluidic immunoassay with a lower limit of detection (LoD) of 3.2 ng/ml PSA in serum samples (Adel Ahmed and Azzazy, 2013). Most smartphone detection systems reported so far are based on colorimetric detection (Oncescu et al., 2013). Although colorimetric detection is usually more cost-effective, easy-to-use and rapid (Lin et al., 2010), fluorescence detection should present higher sensitivity for quantitative POC diagnostics, allowing low analyte cut off values in small sample volumes which is vital for many clinical biomarkers. Smartphone fluorescence detection has been reported in some bioassays, such as quantification of albumin using a dye based assay (Coskun et al., 2013), in a lateral flow assay (Lee et al., 2013) and finally to detect bacteria using a lateral flow assay with fluorescence nanoparticles (Rajendran et al., 2014). However, smartphone fluorescence detection has not yet been reported in sandwich ELISA systems for accurate quantitation of analytes, and this would bring POC microfluidic diagnostics to a new level of portability and sensitivity.

In this study we present a flexible smartphone based colorimetric and fluorescence detection system, termed the MCFphone, capable of detecting PSA from whole blood in the relevant clinical range in 13 min using colorimetric detection and 22 min using fluorescence detection. PSA is the mostly widely used prostate cancer biomarker, and continuous monitoring of PSA levels in patients with prostate cancer is a vital diagnostic tool. PSA blood levels determination in conjugation with digital rectal examination was approved by the Food and Drugs Administration to test asymptomatic men aged 50 year old with a cut off value of 4 ng/ml of PSA (De Angelis et al., 2007; Institute, 2012). Many studies suggested that prostate cancer mortality can be decreased by early detection, and so screening programs have been proposed utilising PSA quantitation in blood. We propose that a quantitative whole blood PSA sandwich assay in a rapid, sensitive, and portable test device would allow POC prostate cancer monitoring and screening even in remote areas of developing countries where laboratory facilities are limited. The MCFphone detection system could easily be combined with the "Lab-in-a-briefcase" assay platform reported recently (Barbosa et al., 2014), replacing the flatbed scanner readout system and increasing portability, flexibility and sensitivity.

2. Materials and methods

2.1. Materials and reagents

Enzymatic chromogenic and fluorescence products 2,3-diaminophenazine (DAP) and fluorescein isothiocyanate (FITC), dimethyl sulfoxide (DMSO), streptavidin alkaline phosphatase, SIGMA-FASTTM OPD (o-Phenylenediamine dihydrochloride) tablets and FDP (fluorescein diphosphate) were sourced from Sigma-Aldrich (Dorset, UK). High sensitivity streptavidin-HRP was supplied by Thermo Scientific (Lutterworth, UK).

Human kallikrein 3/ Prostate Specific Antigen (PSA) ELISA kit was purchased from R&D Systems (Minneapolis, USA). The kit contained a monoclonal mouse Human Kallikrein 3/PSA antibody (capture antibody), a Human Kallikrein 3/PSA polyclonal biotynilated antibody (detection antibody) and recombinant Human Kallikrein 3/PSA (standard). Phosphate buffered solution (PBS) and Bovine Serum Albumin (BSA) were sourced from Sigma Aldrich, Dorset, UK. PBS pH 7.4, 10 mM was used as the main immunoassay buffer. Carbonate buffer 50 mM pH 8 and Tris Buffer 50 mM pH 9.2 were used for fluorescence detection with alkaline phosphatase. The blocking solution consisted in 3% w/v protease-free BSA diluted in PBS buffer. For washings, PBS with 0.05% v/v of Tween-20 (Sigma-Aldrich, Dorset, UK) was used. The whole blood used was obtained from donation system at Loughborough University, Sports Department, and collected into 5 ml tubes with citrate phosphate as anticoagulant.

The MicroCapillary Film (MCF) platform is fabricated from fluorinated ethylene propylene co-polymer (FEP-Teflon®) by melt-extrusion process by Lamina Dielectrics Ltd. (Billinghurst, West Sussex, UK). The number and internal diameter of the microcapillaries is easily controlled by the design of the die and the operational conditions set during the continuous melt-extrusion process. The two primary light sources used include an Auraglow AG166 Blue LED bulb, from Argos UK and an Ultraviolet Mini Lantern UV Fluorescent purchased from Mapplin UK. A 60x magnification attachment for iPhone® 4/4S purchased from Amazon (Slough, Berkshire) and a 50 mm square dichroic additive green filter sourced from Edmund Optics (York, UK).

2.2. MCFphone – system overview

The MCFphone detection system is composed by a 10 bore fluoropolymer MCF strips pre-coated with immobilized capture antibody and blocked with BSA protein (1), smartphone (iPhone® 4S, 8 megapixels camera) (2) integrated magnifying lens (3), light source (blue LED, with peak wavelength of 450 nm for chromogenic detection) (4), or UV black light for fluorescence detection (5), and a dichroic additive green filter (6) for fluorescence detection (Fig. 1A).

The MCF used consisted of a fluoropolymer melt-extruded plastic film with 10 embedded parallel microcapillaries and a mean 200 μm internal diameter. Each strip has 4.5 ± 0.1 mm width and 0.6 ± 0.05 mm depth (Fig. 1B). This platform was first presented as a cost-effective microfluidic immunoassay platform by Edwards et al. (2011); the hydrophobicity of FEP material allows simple yet effective immobilisation of antibodies by passive adsorption on the plastic surface of the microcapillaries, and the transparency of the MCF material results in high signal-to-noise ratios (Edwards et al., 2011) which is fundamental for sensitive signal quantitation.

The MCFphone working principle consists in illuminating the MCF test strip sample with a light source (blue LED for chromogenic detection and UV black light for fluorescence detection) and capturing the signal (digital image) with a smartphone camera attached with a magnifying lens (Fig. 1C). The digital images were then analysed with *Image J* software (NIH, Maryland, USA) for colourimetric or fluorescence signal quantitation.

2.3. PSA sandwich ELISA (enzyme linked immunosorbent assay)

2.3.1. Fabrication of MCF test strips

A solution of 40 μg/ml of Human Kallikrein 3/PSA capture antibody in PBS buffer was aspirated into a 100 cm lengh MCF and incubated for two hours at room temperature in a petri dish covered with a wet tissue to avoid evaporation of solution in the microcapillaries. A 3% BSA solution in PBS buffer was then aspirated and incubated in the MCF for an additional two hours at room temperature to block any additional binding sites in the microcapillaries. The MCF strip was then washed with 0.05% Tween in PBS, and stored in the fridge at 4 °C or used immediatetly. The MFC was then trimmed into 3 cm long test strips and interfaced with a single 1 ml syringe using a short 3 mm i.d.

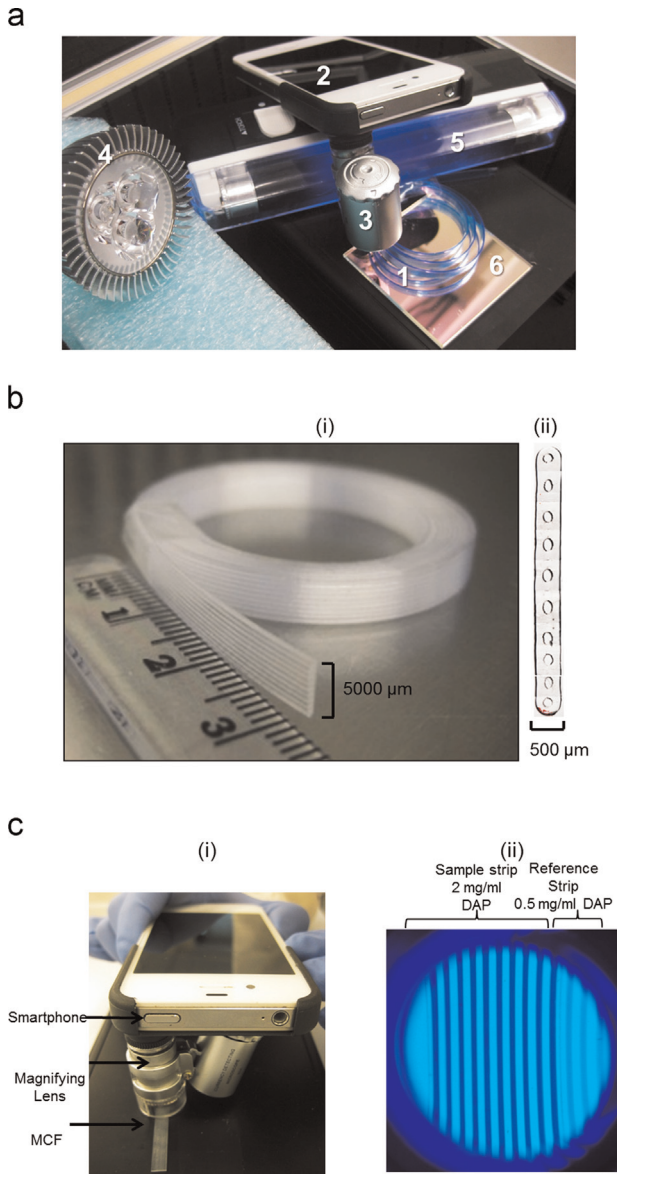

Fig. 1. MCFphone system overview. (A) Main components of MCFphone (1) Microcapillary Film (MCF) (2) Smartphone (3) Magnifying lens (4) Blue LED (5) UV black light for fluorescence detection, light source for chromogenic detection (6) dichroic filter. (B) The Microcapillary Film (MCF), melt extruded from fluorinated-ethylene-propylene (FEP) copolymer (i) 5 m MCF reel (ii) MCF cross section with 10*200 µm embedded in the FEP polymer. (C) MCFphone detection in operation (i) MCFphone components (ii) MCFphone colorimetric image of a sample and a reference strips filled up with 2,3-diaminophenazine (DAP), the product of HRP conversion of o-Phenylenediamine dihydrochloride.

silicon tubing and a plastic clamp or integrated in a 8-channel multi-syringe aspirator; this semi-automatic device allows 8 test strips and a total of 80 capillaries to be filled simultaneously using a set of 1 ml plastic syringes with minimum training as described elsewhere (Barbosa et al., 2014).

2.3.2. PSA sandwich assay

The PSA sandwich assay consisted in pre-loading 150 µl of sample and immunoassay reagents into each well in microplate especially designed to interface the MCF strips. The test strips were then immersed in microplate wells and solutions aspirated sequentialy by manually moving the piston plunger of the disposable syringe as illustrated in Fig. 2A. In all experiments shown in this paper 8 MCF strips were operated simultaneously based on the multi-syringe aspirator, which is capable of runing a 7 point full response curve plus a control or analysing multiple samples simultaneously. From the perspective of end use, it only requires rotating the knob that controls the position of the piston plungers in the array of 1 ml disposable syringes and moving the device along a multiplexed microplate containing all pre-loaded reagents. On each incubation step, about 78 µl of solution are aspirated corresponding to 6 full rotations of the knob. With this design, the total number of steps required to complete the PSA sandwich immunoassay was reduced to 5, and all waste solutions were kept inside the 1 ml disposable syringes throughout the assay. The incubation times used for PSA immunoassay are summarised in Table 1. The concentration of biotinylated detection antibody was 1 µg/ml and PSA recombinant protein standards were diluted in PBS or whole blood where appropriate.

For colorimetric detection, 1 µg/ml solution of high sensitivity streptavidin-HRP was used for enzymatic amplification, and the chromogenic substrate consisted of 4 mg/ml OPD and 1 mg/ml H_2O_2. Smartphone microphotographies of the MCF strips were taken with a iPhone® 4S at 20 sec interval for a total of 2 min after addition of the enzymatic substrate. For fluorescence detection, 2 µg/ml of alkaline phosphatase was diluted in carbonate buffer 50 mM, pH 8, and the fluorescence enzymatic substrate consisted in 0.25 mM solution of fluorescent substrate FDP; the MCF strips were photographed at 60 sec interval for a total of 10 min. On both colourimetric and fluorescence assays, the MCF strips were extensively washed with PBS-Tween before aspirating the enzymatic substrate.

2.4. Colorimetric and fluorescence detection

Accurate quantitation with smartphone cameras demands control of environment light (Oncescu et al., 2013), once the exposure time of the camera is automatically adjusted in response to the amount of light passing through the detection region (Lee et al., 2014). In order to control environmental light during colorimetric detection a small polyethylene box and a blue LED, with peak 450 nm emission (absorption peak for DAP) were set together. The blue LED was placed so that the light penetrated along the length of the channels rather than across them. However, even with the external environment light control, the light passing through the detection region was not always consistent due to different absorbance of the samples. To eliminate the effect of different exposure times of the camera, all smartphone images were taken with a reference MCF strip (Fig. 1B (i)) loaded with the final product of enzymatic conversion; the signal from this strip was then further used for data normalisation as detailed in Section 2.5.

To test for sensitivity of smartphone detection, MCF strips were filled with the chromogenic substance 2, 3-diaminophenazine (DAP, resulting from OPD enzymatic substrate conversion) in 1:2 serial dilution in distilled water, from 0.074 to 9.5 mM and imaged with the MCFphone. Sensitivity of the detection system was calculated by analysing the grey scale in the microphotographs of the MCF strips and adding 3 times the blank standard deviation to the blank value. This gives the minimum absorbance value detectable with the colorimetric setup, therefore setting the sensitivity of the system.

Fluorescence quantitation was carried out in the dark using an Ultraviolet Mini Lantern UV to excite the fluorescence product in the microcapillaries. The UV light was placed under a mask with an aperture sufficient to place both the MCF strip sample and reference. The MCF strips were then overlap with a green dichroic additive filter allowing only the green colour (530 nm) to pass through and be capture by the smartphone placed on the top of the filter.

Sensitivity of fluorescence detection mode was tested by filling

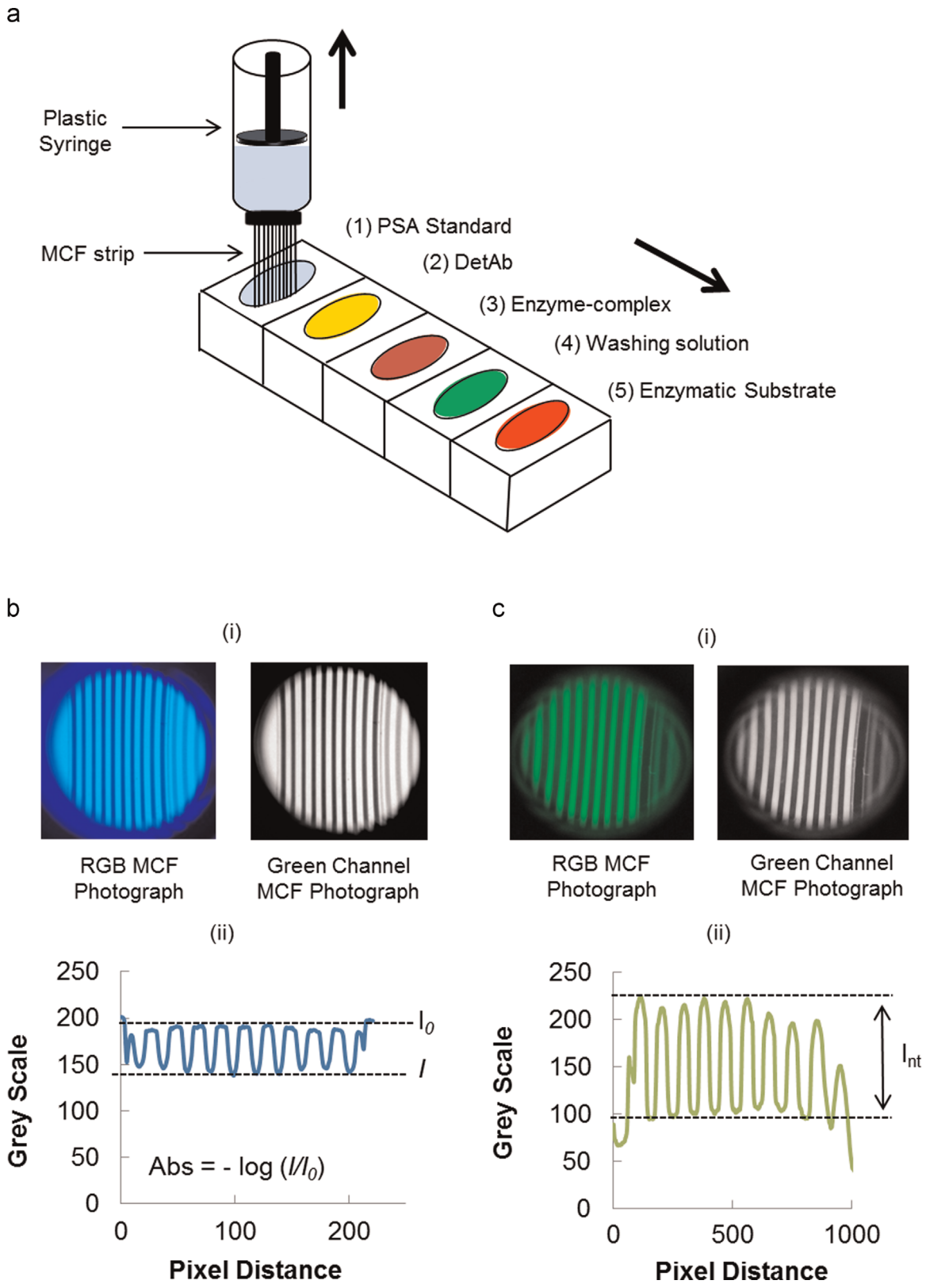

Fig. 2. PSA sandwich assay with MCFphone signal quantitation. (A) PSA sandwich assay steps. (B) Colorimetric signal quantitation (i) RGB and green channel image (1 mg/ml) with reference strip (0.5 mg/ml) of 2,3-diaminophenazine (DAP) (ii) Correspondent grey scale analysis. (C) Fluorescence signal quantitation (i) RGB and green channel image (0.5 mM of fluorescein isothiocyanate, FITC, with reference strip of 0.125 mM) (ii) Correspondent grey scale analysis.

up 8 MCF strips with 1:2 serial dilution of Fluorescein Isothiocyanate (FITC) in distilled water in the 7.8 to 500 μM concentration range. The strips were placed in the dark metal support according to fluorescence MCFphone detection setup and smartphone photographs recorded. In the same way to colorimetric system sensitivity, fluorescence sensitivity was calculated by adding 3 times the blank standard deviation to the blank after image analysis.

Table 1
Incubation times comparison between PSA colorimetric assay and fluorescence assay.

Incubation Times (min)		
	PSA colour assay	PSA fluorescent assay
Sample/PSA standard	2	2
Detection Antibody	5	5
Enzyme-complex	5	5
Enzymatic substrate	1	10
Total Assay Time	13	22

2.5. Image analysis

The iPhone® 4S images were analysed with *ImageJ* software, and consisted in splitting the acquired RGB images into Red, Green and Blue (RGB) channels for both colorimetric and fluorescence detection modes. For colorimetric quantitation, the absorbance (Abs) was calculated from the mean pixel intensity in grey scale in the green channel as shown in Fig. 2B (i) based in Eq. (1), where I_0 is the mean grey scale intensity of the baseline and I is determined based on the difference between the baseline and the maximum grey scale peak height, h as seen in Eq. (2) (Fig. 2C (ii)). The microcapillaries in the MCF have a circular to elliptical geometry, therefore the transmittance images have a minimum grey scale intensity at the centre of the capillaries where the light path distance is maximum. The Abs value presented for each MCF sample studied is a mean Abs value calculated by averaging the whole array of 10 individual microcapillaries in each MCF strip. The Abs value for each strip was further normalised based on the Abs value of the reference strip in order to compensate for the automatic settings of the smartphone camera, yielding the Absorbance ratio, Abs_{ratio} shown in Eq. (3).

$$Abs = -\log_{10}\left(\frac{I}{I_0}\right) \quad (1)$$

$$I = I_0 - h \quad (2)$$

$$Abs\ Ratio = \frac{Abs_{sample,\,i}}{Abs_{ref}} \quad (3)$$

For fluorescence quantitation in the MCFphone, the split green channel image (Fig. 2C (i)) was used to produce a grey scale plot from where the fluorescence intensity, I_{int} was measured for each individual microcapillary in the MCF strips (Fig. 2C (ii)). The intensity of the peaks for each capillary was then normalised by the mean intensity peak of the reference strip, $I_{int,ref}$ thus eliminating variability of camera exposure settings. Consequently, fluorescence signal is presented as an average of fluorescence intensity ratio (fluorescence ratio) of the 10 capillaries in each MCF strip (Eq. (4)).

$$Fluorescence\ ratio = \frac{I_{int,sample}}{I_{int,ref}} \quad (4)$$

The LoD was determined as the minimum concentration yielding a signal higher than the blank value plus 3 times the standard deviation of the blank. When appropriate this value was calculate using a 4PL (4 parameter logistic model) best-fitted to the experimental response curve.

2.6. Recovery PSA experiments from whole blood samples

%Recovery was calculated for three different anticoagulated fresh whole blood samples (samples S1, S2 and S3) spiked with 0.74, 6.7 and 60 ng/ml of PSA recombinant protein. Fluorescence intensity was then determined in the MCFphone for the different blood samples using procedure described in Section 2.5. The % Recovery was calculated based on the ratio of the Abs or fluorescence signal in assay buffer to the signal in the blood sample in percentage basis. A iPhone® 5s was used to microphotograph the MCF test strips.

3. Results and discussion

3.1. Sensitivity of MCFphone for detection of chromogenic and chemifluorescence substrates

Optical detection based on simple and cost-effective technologies like a smartphone demands specific optical properties from the assay platform to be able to use small changes in transmitted light intensity for analyte quantitation. The MCF fluoropolymer has special optical properties, such as high degree of transparency obtained by a matched refractive index of FEP material with an aqueous sample, combined with the film flat geometry that minimises light diffraction, reflection or scattering (Edwards et al., 2011). This favours sensitive immunoassays that combined with short diffusion distances yields a powerful miniaturised and portable POC immunoassay concept.

Colorimetric detection is inherently less sensitive than fluorescence, due to fundamental limitations of colorimetry itself. From one hand, in order to measure low concentrations of a chromogen, small differences in intensity must be measured at high light intensity, which limits the LoD. On the other hand, relationship between optical Abs and intensity of transmitted light is logarithmic. Therefore, at high chromogen concentrations relatively large differences in optical absorbance correspond to small differences in the intensity of unabsorbed light, which usually corresponds to narrow dynamic range for immunoassays (Gosling, 1996). However, colorimetric detection is still widely used in immunoassays, offering speed, simplicity, well established assay chemistry and high quality reagents, and widespread availability of cost-effective readers. For this reason researchers still focused in finding new ways to increase the performance of colorimetric detection, for example through enzymatic amplification systems (Brooks et al., 1991; Gao et al., 2014). Besides being inherently less sensitive, colorimetric detection sensitivity can also be limited by the working range of the instrument.

In our recent study, we have measured PSA in biological samples in less than 15 min using a flatbed scanner (Barbosa et al., 2014). In this study we aimed turning the "Lab-in-a briefcase" power-free, by using a portable detection system. Understanding the sensitivity and dynamic range of colorimetric measurement using the MCFphone setup is therefore fundamentally important, since performance for absorbance measurement will limit the working range and sensitivity of MCF immunoassays. As a benchmark, a spectrophotometer was used to measure a range of concentrations of DAP, the chromogenic product of enzymatic conversion of the substrate OPD commonly used in enzyme-linked immunosorbent assays (ELISA). At the peak absorption of 425 nm (Fig. 3A), the spectrophotometer has a LoD of < 2.3 μM of DAP (Fig. 3B). In contrast, the MCFphone sensitivity for DAP measurement was 148 μM (Fig. 3C), i.e. 2 orders of magnitude less sensitive than a sophisticated spectrophotometer with a 1 cm thick cuvette. This drop in sensitivity can be accounted for by the broad wavelengths measured of RGB system, compared to the specific narrow wavelength selection in a spectrophotometer. Also, the MCFphone presents a 50-fold reduction in the light path when compared to the spectrophotometer cuvette, which reduces the absolute absorbance for any concentration of a chromogen.

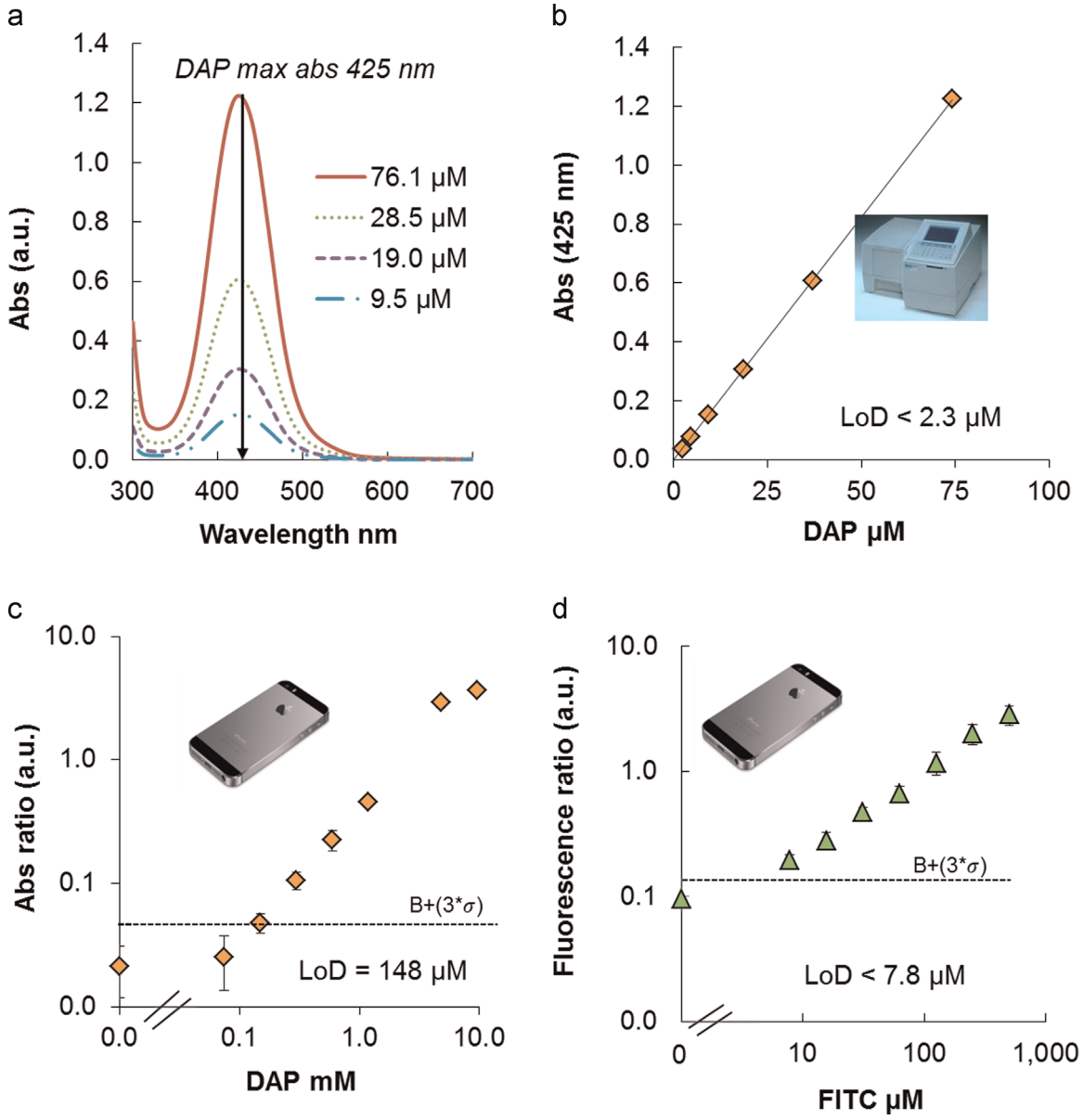

Fig. 3. Sensitivity of MCFphone for colorimetric and fluorescence detection. (A) Absorption spectrum of 2,3-diaminophenazine (DAP). (B) Sensitivity of spectrophotometer DAP relative. (C) MCFphone chromogenic sensitity determination (DAP serial dilution). (D) MCFphone fluorescence sensitivity determination (fluorescein isothiocyanate, FITC serial dilution).

Sophisticated benchtop instruments allow higher sensitivity measurement for colorimetric substrates, however they are not suitable for POC. Clinical relevant sensitivities from colorimetric portable detection system must therefore be achieved by assay optimisation, for example using enzymatic amplification, as previously observed in ELISA systems, and as demonstrated in the PSA assay results presented in this paper.

Fluorescence detection systems are characteristically more sensitive once they are measured relatively to an absence of light (Gosling, 1996). The MCF phone fluorescence calibration curve, presented a dynamic range of 7.8–500 µM for FITC and a LoD below 7.8 µM (Fig. 3D). The fluorescence detection system selected for further study with PSA immunoassays uses UV light which emits radiation between 340 and 400 nm to excite fluorescein to emit green light at 530 nm. Although this reduces the absorbance efficiency compared to excitation at the peak absorbance of around 480 nm, this system exploits a large stokes shift resulting in a large increase from excitation to emission wavelength, thus eliminating the interference of the incident light with the image acquired by the smartphone which is very insensitive to UV light. This system does not require the use of an expensive bandpass filter, further increasing the simplicity of the optics. Combined with a good quantum efficiency fluorescein is an attractive substrate (Weeks and Kricka, 2013).

The MCFphone achieved similar sensitivity with fluorescence detection to absorbance measurement in the bench-top spectrophotometer, allowing sensitive and portable measurement and justifying the use of fluorescence detection in microfluidic immunoassays (Darain et al., 2009; Lin et al., 2010). The MCFphone is a flexible detection system, with a choice of rapid colour detection or sensitive fluorescence detection that can be selected to suit different applications.

3.2. *Effect of detection mode on sensitivity of PSA sandwich ELISA in*

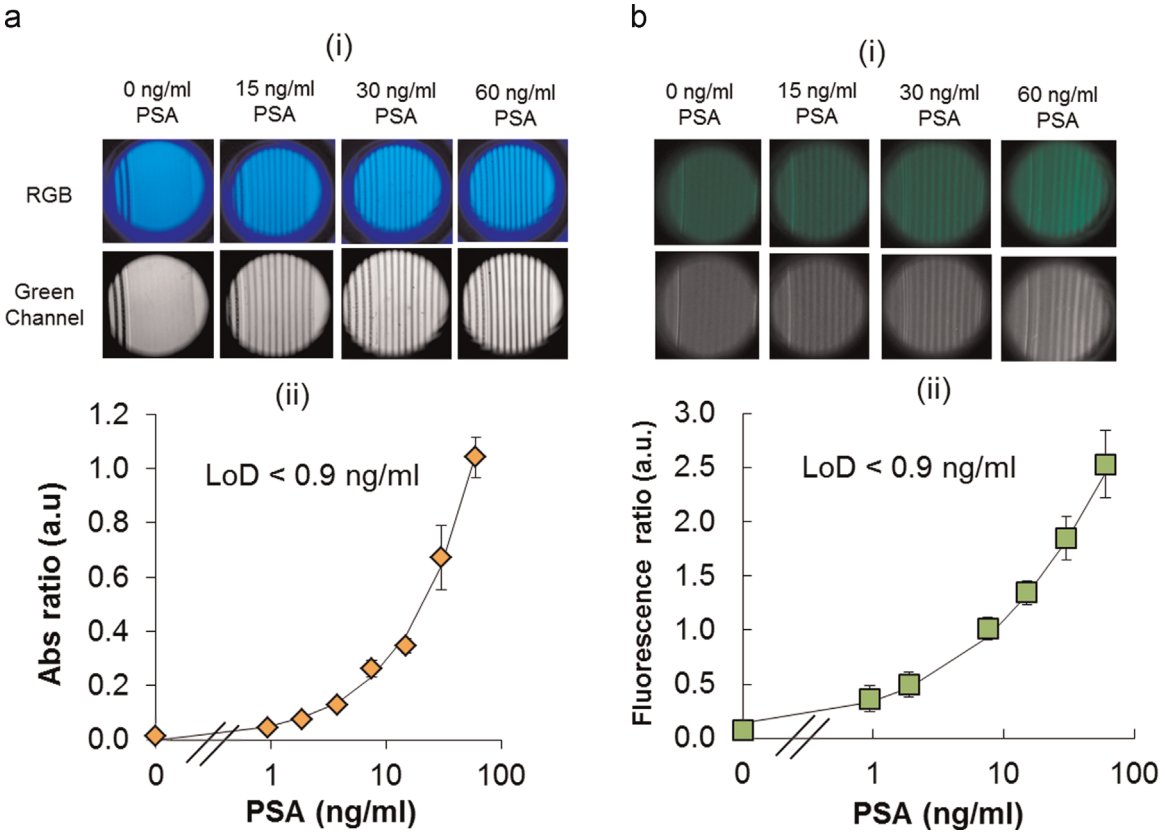

Fig. 4. PSA full response curves in buffer, with MCFphone colorimetric and fluorescence systems. (A) PSA MCFphone colorimetric quantitation: (i) RGB and green channel digital images, and (ii) full response curve, showing a lower limit of detection, LoD < 0.9 ng/ml PSA. (B) PSA MCFphone fluorescence quantitation: (i) RGB and green channel digital images, and (ii) full response curve with LoD clearly below 0.9 ng/ml of PSA.

the MCFphone

Enzymes are the most versatile and common group of labelling substances used in immunoassays (Gosling, 1996). Macromolecules are measurable at very low concentrations typically in the picomolar to femtomolar range by utilising its catalytic properties to generate coloured, fluorescent, or luminescent compounds from a neutral substrate. A single enzyme molecule may cause the conversion of 10^7 molecules of substrate per minute, increasing the strength of the signal and therefore the sensitivity by a million-fold when compared to a label that produces just a signal event (Weeks and Kricka, 2013). When enzymes are associated with antibodies in sandwich immunoassays, the most specific and sensitive type of heterogeneous immunoassays (Christopoulos, 1996), a powerful analytical tool is produced.

Although the colorimetric substrate measurement in the MCFphone was about 2 orders of magnitude less sensitive than the benchtop spectrophotometer, this system was still able to quantify PSA in its clinical relevant range (i.e. a clinical threshold of >4 ng/ml requiring further examination) with LoD of 0.9 ng/ml PSA (Fig. 4A) in 13 min total assay time (Table 1). Reaction time is important for POC applications, where quick tests are required.

In conventional microtiter plate based ELISA high sensitivity is achieved using long incubation times and long assay times, however kinetic studies in MCF platform showed that is possible to achieve high sensitivity with short incubation times and that long incubation times will actually increase the background decreasing sensitivity. Therefore, short incubation times favoured higher signal-to-noise-ratios in the PSA immunoassay.

Despite previous optimisation of PSA assay, the best sensitivity was achieved by combining a sandwich heterogeneous assay format with a horseradish peroxidase (HRP) label. HRP has a high turnover number and due to short diffusion distance of capillaries it was possible to increase the OPD substrate concentration and optimise the molar ratio of OPD to H_2O_2 contributing to faster and higher signal amplification and compensating for the lower sensitivity of colorimetric MCFphone readout.

Fluorescence measurement using the MCFphone gave greater sensitivity than colorimetric, achieving similar LoD to the benchtop spectophometer. This increased sensitivity resulted in even greater assay sensitivity when a full PSA response curve was tested (Fig. 4B) and a LoD of 0.08 ng/ml PSA was achieved. The increased sensitivity of the assay can be attributed to fluorescence detection combined with other factors including the alkaline phosphatase label that like HRP has high enzymatic turnover and the ability of the fluorogenic product (fluorescein) to absorb UV radiation around 380 nm and emit green radiation around 530 nm, which was enhanced with the use of the dichroic additive green filter.

Although fluorescence detection increased sensitivity, the fluorogenic substrate incubation time was longer than the chromogenic substrate incubation time (Table 1), due to differences in reaction rates of the two enzymes. Further optimisation may be required to reduce the overall assay time, and previous work with HRP has demonstrated the improvements to assay time and sensitivity that are possible with systematic assay optimisation in MCF immunoassays (Barbosa et al., 2014).

Similar studies measured PSA with a microfluidic device incorporating colorimetric smartphone detection, and reported 3.2 ng/ml PSA as the LoD in 30 min total assay time using magnetic microbeads (Adel Ahmed and Azzazy, 2013), thus a 3.5 lower sensitivity for double of the assay time compared to the MCFphone.

Fluorescent measurements performed with the immuno-pillar microfluidic platform reported a LoD of 0.1 ng/ml in 12 min total

assay time, which means similar sensitivity, but double of time for fluorescence detection on MCFphone. However, it is important to emphasise that the detection equipment used to quantify PSA in the immuno-pillar platform was an inverted fluorescence microscope, which is not portable, cost-effective or power-free, therefore not suitable for POC applications (Ikami et al., 2010). An immunochromatographic test integrated with a Laser Fluorescence Scanner was able to detect PSA in biological samples with a lower limit of detection of 0.02 ng/ml PSA in 15 min total assay time (Oh et al., 2009). This assay was able to obtain better sensitivity than the MCFphone in less time. Nevertheless, lateral flow tests have certain limitation compared with microfluidic devices, for example they lack the possibility to perform calibration curves in simultaneous with the samples (Posthuma-Trumpie et al., 2009), which can be an important feature for biomarker quantitation at POC. Besides the drawbacks of lateral flow technology, the Laser Fluorescence Scanner is not as flexible as a smartphone, once it can be used for colorimetric detection, for data analysis and for results communication all in one single power-free and portable device, which is quickly becoming a cost-effective equipment. In addition, this is the first time to our knowledge that a smartphone is used to quantify fluorescence in a sandwich ELISA assay.

3.3. PSA measurement in whole Blood samples

In POC testing, sample processing needs to be minimised or eliminated to speed up the diagnostic process (von Lode, 2005) so whole blood samples are preferred. However, whole blood has one of the most complex matrices, with interference possible due to serum proteins that can bind non-specifically to analytes, antibodies or surfaces (Chiu et al., 2010). This biological fluid also presents a high viscosity that can alter the binding efficiency (Morgan et al., 1998) and the diffusion time of the analyte to the immobilised antibody. Significant work has been published describing methods to overcome the effect of biological matrices in order to achieve robust assay sensitivity and reproducibility in other laboratory and POC assay platforms (Chiu et al., 2010). Matrix effects are system specific, depending on matrix type,

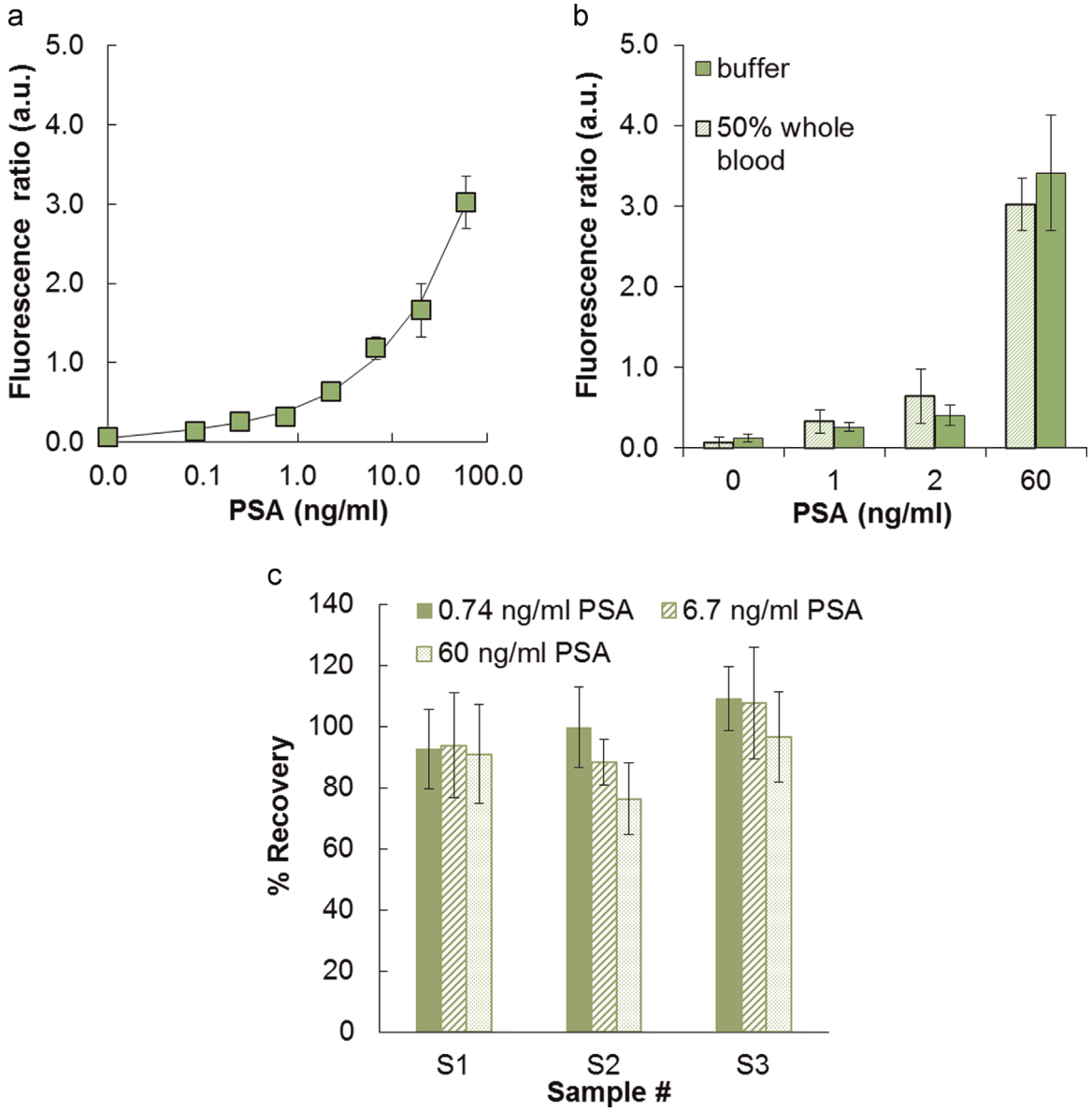

Fig. 5. Smartphone fluorescence detection of PSA in the MCF in whole blood. (A) Full response curve using 1:3 dilution series. (B) Comparison between PSA sandwich assay in buffer and 50% whole blood. (C) %Recovery for 3 different blood samples from female donors spiked with 3 different concentrations of recombinant protein, 0.74, 6.7 and 60 ng/ml.

Table 2
Sensitivity comparison between MCFphone colorimetric and fluorescence PSA assay.

	Lowest PSA conc. tested (ng/ml)	Data correlation with 4PL model (R^2)	LoD (ng/ml)	Precision
PSA colour assay	0.9	0.9979	0.40	7% at 15 ng/ml PSA
PSA fluorescent assay	0.9	0.9933	0.08	8% at 15 ng/ml PSA
PSA fluorescent assay in whole blood	0.08	0.9978	0.04	11% at 2.2 ng/ml PSA

detection method, antibody system and platform. The matrix effect has already been reported by our research group for sandwich ELISA detection of PSA in whole blood in the MCF platform using a flatbed scanner readout system (Barbosa et al., 2014). From these studies we concluded that PSA could be accurately quantified in whole blood samples in the MCF platform. However, non-diluted whole blood matrices did show lower signals for the same incubation times as buffer matrices. This signal reduction could be overcome either by dilution or by extended sample incubation times. As the aim of this work is to create a rapid POC diagnostic system, sample dilution was chosen here instead of extended sample incubation times to maintain the sensitivity and reproducibility of PSA assay in biological samples and restrict total assay time. Sample dilution is often used in serology and does not represent an additional step in the assays, as the sample wells can be provided with diluent buffer.

In this study, the MCFphone fluorescence detection system was used to quantify PSA from diluted whole blood (Fig. 5). The sensitivity calculated and assay signal were in the same order of magnitude (Fig. 5A and Table 2) when compared to the assay performed in buffer across all the PSA assay range (Fig. 5B). Further experiment using whole blood from 3 additional patients showed a %Recovery very consistent when spiked with PSA concentration in the low linear range (0.74 ng/ml), middle range (6.7 ng/ml) and higher linear range (60.0 ng/ml). In all cases, % Recovery values calculated for the different whole blood samples remained within 80–120%, which is regarded as the desirable range, with the exception of S2 spiked with 60.0 ng/ml of PSA which returned 76.3%. This ultimately demonstrates the MCFphone as an efficient PSA measurement system from fresh anticoagulated whole blood samples, simplifying sample preparation required for clinical testing. Future studies will aim understanding blood matrix effect on variability between samples, and test the MCFphone with patients diagnosed with prostate cancer.

4. Conclusions

The MCFphone is a flexible, power-free and portable immunoassay detection system capable of rapid colorimetric detection and sensitive fluorescence detection from whole blood samples. In this present study, the MCFphone was able to detect and quantify PSA within the dynamic range of 0.9 to 60 ng/ml PSA in 13 min, using colorimetric detection and within 0.08 ng/ml to 60 ng/ml of PSA, using fluorescence detection from whole blood samples. Considering the PSA clinical range of 4 ng/ml for prostate biopsy, this device can provide reliable measurements for prostate cancer screening and detection in remote areas outside laboratory facilities. Further improvements in fluorescence sensitivity could be obtained with optimisation of enzymatic reaction, a more robust and intense UV light source and filters, as well as higher resolution smartphone cameras. Automation of multistep ELISA and further integration and miniaturisation of all fluidic and optoelectronics components will addressed in future publications with the MCFphone concept.

Conflict of interest

ADE and NMR are shareholders and directors of Capillary Film Technology Ltd (CFT), an UK based start-up developing fluoropolymer MCF for applications in life sciences and molecular diagnostics.

Acknowledgements

The authors are grateful to Patrick Hester from Lamina Dielectrics Ltd for providing the MCF material, and to Cristiana Ferreira and James Mcveigh for assistance with iPhone® imaging of the test strips. This work was co-funded by Capillary Film Technology Ltd, Loughborough University and EPSRC (grant code EP/L013983/1).

References

Adel Ahmed, H., Azzazy, H.M.E., 2013. Power-free chip enzyme immunoassay for detection of prostate specific antigen (PSA) in serum. Biosens. Bioelectron. 49, 478–484. http://dx.doi.org/10.1016/j.bios.2013.05.058.

Altintas, Z., Fakanya, W.M., Tothill, I.E., 2014. Cardiovascular disease detection using bio-sensing techniques. Talanta 128, 177–186. http://dx.doi.org/10.1016/j.talanta.2014.04.060.

Barbosa, A.I., Castanheira, A.P., Edwards, A.D., Reis, N.M., 2014. A lab-in-a-briefcase for rapid prostate specific antigen (PSA) screening from whole blood. Lab Chip, 2918–2928. http://dx.doi.org/10.1039/c4lc00464g.

Brooks, J.L., Mirhabibollahi, B., Kroll, R.G., 1991. Increased sensitivity of an enzyme-amplified colorimetric immunoassay for protein A-bearing Staphylococcus aureus in foods. J. Immunol. Methods 140, 79–84. http://dx.doi.org/10.1016/0022-1759(91)90128-3.

Chiu, M.L., Lawi, W., Snyder, S.T., Wong, P.K., Liao, J.C., Gau, V., 2010. Matrix Effects—A Challenge Toward Automation of Molecular Analysis. J. Assoc. Lab. Autom. 15, 233–242. http://dx.doi.org/10.1016/j.jala.2010.02.001.

Diamandis, E.P., Christopoulos, T.K., 1996. Immunoassay. Academic Press, San Diego/London.

Coelho, F.R., Martins, J.O., 2012. Diagnostic methods in sepsis: the need of speed. Rev. Assoc. Med. Bras. 58, 498–504. http://dx.doi.org/10.1590/S0104-42302012000400024.

Coskun, A.F., Nagi, R., Sadeghi, K., Phillips, S., Ozcan, A., 2013. Albumin testing in urine using a smart-phone. Lab Chip 13, 4231–4238. http://dx.doi.org/10.1039/c3lc50785h.

Darain, F., Yager, P., Gan, K.L., Tjin, S.C., 2009. On-chip detection of myoglobin based on fluorescence. Biosens. Bioelectron. 24, 1744–1750. http://dx.doi.org/10.1016/j.bios.2008.09.004.

De Angelis, G., Rittenhouse, H.G., Mikolajczyk, S.D., Blair Shamel, L., Semjonow, A., 2007. Twenty years of PSA: From prostate antigen to tumor marker. Rev. Urol. 9, 113–123.

Edwards, A.D., Reis, N.M., Slater, N.K.H., Mackley, M.R., 2011. A simple device for multiplex ELISA made from melt-extruded plastic microcapillary film. Lab Chip 11, 4267–4273. http://dx.doi.org/10.1039/C0LC00357C.

Erickson, D., O'Dell, D., Jiang, L., Oncescu, V., Gumus, A., Lee, S., Mancuso, M., Mehta, S., 2014. Smartphone technology can be transformative to the deployment of lab-on-chip diagnostics. Lab Chip 14, 3159–3164. http://dx.doi.org/10.1039/c4lc00142g.

Gao, Z., Hou, L., Xu, M., Tang, D., 2014. Enhanced colorimetric immunoassay accompanying with enzyme cascade amplification strategy for ultrasensitive detection of low-abundance protein. Sci. Rep. 4, 3966. http://dx.doi.org/10.1038/srep03966.

Gervais, L., de Rooij, N., Delamarche, E., 2011. Microfluidic chips for point-of-care immunodiagnostics. Adv. Mater. 23, H151–H176. http://dx.doi.org/10.1002/adma.201100464.

Gervais, L., Delamarche, E., 2009. Toward one-step point-of-care immunodiagnostics using capillary-driven microfluidics and PDMS substrates. Lab Chip 9, 3330–3337. http://dx.doi.org/10.1039/b906523g.

Gosling, J.P., 1996. Immunoassay. Elsevier, United States of America, http://dx.doi.

org/10.1016/B978-012214730-2/50014-5, ISBN-13: 978-0-12-214730-2, ISBN: 0-12-214730-8.
Ikami, M., Kawakami, A., Kakuta, M., Okamoto, Y., Kaji, N., Tokeshi, M., Baba, Y., 2010. Immuno-pillar chip: a new platform for rapid and easy-to-use immunoassay. Lab Chip 10, 3335–3340. http://dx.doi.org/10.1039/c0lc00241k.
National Cancer Institute, 2012. Prostate-Specific Antigen (PSA) Test [WWW Document]. ⟨http://www.cancer.gov/cancertopics/factsheet/detection/PSA⟩ (accessed August 2014).
Lee, L., Nordman, E., Johnson, M., Oldham, M., 2013. A low-cost, high-performance system for fluorescence lateral flow assays. Biosensors 3, 360–373. http://dx.doi.org/10.3390/bios3040360.
Lee, S., Oncescu, V., Mancuso, M., Mehta, S., Erickson, D., 2014. A smartphone platform for the quantification of vitamin D levels. Lab Chip 14, 1437–1442. http://dx.doi.org/10.1039/c3lc51375k.
Lin, C.-C., Wang, J.-H., Wu, H.-W., Lee, G.-B., 2010. Microfluidic Immunoassays. J. Assoc. Lab. Autom. 15, 253–274. http://dx.doi.org/10.1016/j.jala.2010.01.013.
Loeb, S., Catalona, W.J., 2007. Prostate-specific antigen in clinical practice. Cancer Lett. 249, 30–39. http://dx.doi.org/10.1016/j.canlet.2006.12.022.
Morgan, C.L., Newman, D.J., Burrin, M., Price, C.P., J., 1998. The matrix effects on kinetic rate constants of antibody–antigen interactions reflect solvent viscosity. J. Immunol. Methods 217, 51–60. http://dx.doi.org/10.1016/S0022-1759(98)00092-1.
Myers, F.B., Lee, L.P., 2008. Innovations in optical microfluidic technologies for point-of-care diagnostics. Lab. Chip 8, 2015–2031. http://dx.doi.org/10.1039/b812343h.
Oh, S.W., Kim, Y.M., Kim, H.J., Kim, S.J., Cho, J.-S., Choi, E.Y., 2009. Point-of-care fluorescence immunoassay for prostate specific antigen. Clin. Chim. Acta 406, 18–22. http://dx.doi.org/10.1016/j.cca.2009.04.013.
Oncescu, V., O'Dell, D., Erickson, D., 2013. Smartphone based health accessory for colorimetric detection of biomarkers in sweat and saliva. Lab Chip 13, 3232–3238. http://dx.doi.org/10.1039/c3lc50431j.
Park, J., Sunkara, V., Kim, T.-H., Hwang, H., Cho, Y.-K., 2012. Lab-on-a-disc for fully integrated multiplex immunoassays. Anal. Chem. 84, 2133–2140. http://dx.doi.org/10.1021/ac203163u.
Park, T.S., Li, W., McCracken, K.E., Yoon, J.-Y., 2013. Smartphone quantifies Salmonella from paper microfluidics. Lab Chip 13, 4832–4840. http://dx.doi.org/10.1039/c3lc50976a.
Pierce, M.C., Weigum, S.E., Jaslove, J.M., Richards-Kortum, R., Tkaczyk, T.S., 2014. Optical systems for point-of-care diagnostic instrumentation: analysis of imaging performance and cost. Ann. Biomed. Eng. 42, 231–240. http://dx.doi.org/10.1007/s10439-013-0918-z.
Posthuma-Trumpie, G. a, Korf, J., van Amerongen, A., 2009. Lateral flow (immuno) assay: its strengths, weaknesses, opportunities and threats. A literature survey. Anal. Bioanal. Chem. 393, 569–582. http://dx.doi.org/10.1007/s00216-008-2287-2.
Rajendran, V.K., Bakthavathsalam, P., Jaffar Ali, B.M., 2014. Smartphone based bacterial detection using biofunctionalized fluorescent nanoparticles. Microchim. Acta 181, 1815–1821. http://dx.doi.org/10.1007/s00604-014-1242-5.
Shen, L., Hagen, J. a, Papautsky, I., 2012. Point-of-care colorimetric detection with a smartphone. Lab Chip 12, 4240–4243. http://dx.doi.org/10.1039/c2lc40741h.
Von Lode, P., 2005. Point-of-care immunotesting: approaching the analytical performance of central laboratory methods. Clin. Biochem. 38, 591–606. http://dx.doi.org/10.1016/j.clinbiochem.2005.03.008.
Weeks, I., Kricka, L.J., 2013. The immunoassay handbook. Elsevierhttp://dx.doi.org/10.1016/B978-0-08-097037-0.00018-X.
Zhang, B., Cai, F.F., Zhong, X.Y., 2011. An overview of biomarkers for the ovarian cancer diagnosis. Eur. J. Obstet. Gynecol. Reprod. Biol. 158, 119–123. http://dx.doi.org/10.1016/j.ejogrb.2011.04.023.
Zhu, H., Isikman, S.O., Mudanyali, O., Greenbaum, A., Ozcan, A., 2013. Optical imaging techniques for point-of-care diagnostics. Lab Chip 13, 51–67. http://dx.doi.org/10.1039/c2lc40864c.